PHOTOSYNTHETICA 17 (2): 198—203, 1983

Chloroplast Crystalloids and Other Alterations in Response to Lichen Substances*

C. ASCASO**, I. ORUS*** and P. ESTÉVEZ***

*Instituto de Edafología y Biología Vegetal, Serrano no. 115 Dpd°, Madrid-6, Spain***
*Cátedra de Fisiología Vegetal, Universidad Complutense de Madrid, Spain****

Abstract

Many lichen phenolics act as allelopathic substances, lowering germination and growth rates of higher plants or even promoting their decay. Physiological observations previously made on the effect of *Evernia prunastri* (L.) Ach. lichen compounds on *Quercus rotundifolia* Lam. explants were correlated in this work with the ultrastructural alterations in chloroplasts. The degenerative changes included the swelling and collapse of granal and agranal thylakoids, with a peripheric display of lamellae and an increase in the number and size of plastoglobuli, though not as high as described elsewhere. Besides these effects, that are in line with the pattern of breakdown of chloroplasts of leaves senescing under natural conditions, bundles of fibrillar formations were found in the stroma. Transverse sections through these stromacentre-like formations showed their paracrystalline structure, made of hexagonal subunits with an electron-transparent core and a dense outline, the centre-to-centre spacing was 13 nm.

Under natural conditions it is easy to find higher plant communities showing clear signs of damage caused by neighbouring lichens, *e.g.* a decrease in the growth rate of *Pinus sylvestris* (Brown and Mikola 1974), an inhibition of tea shoots development (Asahina and Kurokawa 1952), or a partial or complete defoliation of the phorophyte in the wild holm-oak woods of Central Spain by an extensive development of mainly *Evernia prunastri* populations.

The toxicity of lichen phenolic substances has been repeatedly demonstrated under laboratory conditions (Follman and Peters 1966, Pyatt 1967, Ramaut and Thonar 1971a,b, Dauriac and Rondon 1976), and it may, in some instances, be connected with a lowered photosynthetic rate. Estévez and Vicente (1976) showed that these compounds can regulate phycobiont's metabolism acting upon the photosynthetic process, and Orús *et al.* (1981) have proved that *E. prunastri* lichen substances, when added to the incubation medium of *Quercus rotundifolia* explants, inhibited the Photosystem 2 electron flow in *Quercus* chloroplasts; chelation by the lichen substances of the thylakoidal manganese was responsible for such inhibition.

Manganese not only plays a functional role in photosynthesis, but it also has a main structural importance (Teichler-Zallen 1969): under its deficiency, chloroplasts became light-sensitive and their lamellae disorganized.

The ultrastructure of chloroplasts is certainly affected by any kind of stress when the plant has been unable to adapt itself to a new situation, and the degenerative alterations follow a pattern similar to that of normally senescing leaves, whatever the cause of stress is, *i.e.* a viral or fungal infection (Manocha and Shaw 1966), mineral deficiencies (Vesk *et al.* 1966), *etc.* These changes can be resumed as thylakoids swelling and further disintegration, gradual decrease of ribosome population and increase in the number and size of plastoglobuli, together with a marked disorganization of the chloroplast ultrastructure. Some stress situations can promote the presence

* *Received* 26 April 1982.

of crystalline or semicrystalline structures in the stroma: *e.g.* treatment with air pollutants (Thomson *et al.* 1965, 1966, Dolzmann and Ullrich 1966), freezing (Staehelin 1967) or tissue dehydration (Shumway *et al.* 1967, Wrischer 1973). Diaz-Ruiz (1975) related their presence in cytoplasmic vacuoles to growing under nutrient deficiency. Chloroplast crystals may also appear in plants growing under normal conditions, and in some of these cases their formation has been explained by an increased protein synthesis (Wrischer 1973) or by rearranging proteins of disorganized structures.

Having in mind the close relation between structure and function in the chloroplast, the chelating action of lichen substances on thylakoidal manganese and the parallel decay on photosynthetic activity, the authors have studied the ultrastructural modifications on chloroplasts of *Q. rotundifolia* explants treated with *E. prunastri* phenols.

MATERIAL AND METHODS

The chloroplasts were studied in one year-old apical and medial leaves of explants from *Quercus rotundifolia* Lam. collected from El Pardo (Madrid) holm-oak wood.

The lichen substances, the toxicity of which was to be tested, were isolated from the thallus of *Evernia prunastri* (L.) Ach., collected from Hayedo de Montejo (Madrid), by acetone washings and further drying to vacuum, according to Vicente (1975). The quality of extraction and the presence of the four lichen substances of *E. prunastri* (evernic acid, usnic acid, atranorin and chloroatranorin) was checked by thin-layer chromatography (Santesson 1965).

The explants were incubated for one week with 14 $\mu g\ cm^{-3}$ of these phenols dissolved in 1 mM $NaHCO_3$. Control explants, not treated with the lichen extract, were studied at the same time.

Leaf pieces of about 1 mm^2 were fixed in 3.25% glutaraldehyde in 0.1 M Sorensen buffer, pH 7.1, washed in buffer and post-fixed in 1% osmium tetroxide. Dehydration was in graded series of acetone, with saturating uranyl acetate on the 70% step. Samples were embedded in the medium of Spurr (1969). Ultra-thin sections were obtained with an *OmU2 Reichert Ultratome.* Sections were stained with lead citrate according to Reynolds (1963) and examined in a *Philips EM 300* electron microscope.

Acknowledgements: We wish to thank Mr. F. Pinto and Mrs. T. Carnota for technical assistance. Financial support was provided by the Comision Asesora de Ciencia y Tecnologia, Proyecto N° 3768—79.

RESULTS

Just the macroscopic observation of the explants showed that sooner or later the incubation with the lichen substances had a negative effect on *Q. rotundifolia* leaves, where chlorosis and desiccation symptoms appeared. This situation was in agreement with the ultrastructural observations in chloroplasts, where the phenolics caused a high disorganization while control chloroplasts (Fig. 1 *a*) looked normal.

One of the most noticeable alterations (Fig. 1 *b* to *d*) was the irregular aspect of the chloroplast stroma and grana lamellae with a frequent loss or breakdown of granal structure. There was a general decrease of grana concentration and now and then discontinuities were seen in the stacks because of the swelling or breakdown of lamellae. The wide-spread lamellae looked as if they were going to disappear, and very often they swelled and collapsed. (Some of these swelling thylakoids have been indicated by black arrows in Figs. 1 *c* and 2 *a*.)

A peripherical display of the lamellae was usually found (Figs. 1 *b* to *d*, 2 *a*) and in these cases the main part of the stroma was occupied by stromacentre-like structures (Figs. 1 *b*, *d*) and by semi-crystalline bodies without a surrounding membrane (Figs. 1 *b*, *c* and 2 *a*); at higher magnification (Fig. 2*b*) they appeared as arrays of hexagonal units and periodically ordered packing, arranged in honeycomb fashion. The size of the hexagonal subunits was 10–11 nm. With the fixation method employed, they appeared included in a dense matrix and made of an electrontransparent outline and a dense core.

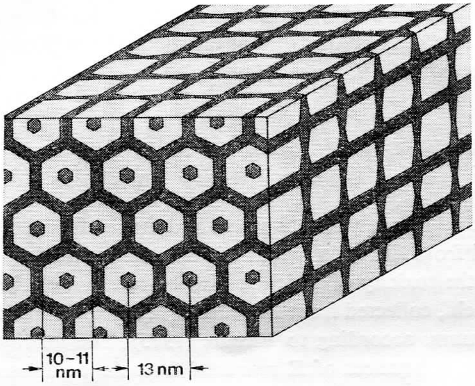

Fig. 3. Schematic model of the authors' interpretation of stromacentre-like structures and semi-crystalline bodies. The stromacentre-like formations and the semi-crystalline bodies are very likely the longitudinal and transverse sections of the same structure.

Having in mind their size and location, these semicrystalline bodies may very likely correspond to transverse sections of the stromacentre-like structures we described above, that appear as parallel lines, alternating dense and electron-transparent bands (for a schematic model of the authors' interpretation of the relation between both structures see Fig. 3).

The number and size of the plastoglobuli has also been increased by the lichen substances (Fig. 1 *d*).

The analyses for viral infection were always negative and thus virus infection could not be responsible for the presence of described structures in plants treated with lichen substances.

DISCUSSION

The stress situation resulting from the treatment with lichen substances is concomitant with ultrastructural alterations in chloroplasts of *Quercus* branches. The pattern of changes follows the characteristic sequence of senescence processes, as described by Butler and Simon (1971). The most easily detectable change is the swelling and disintegration of thylakoids, that might be related to manganese depletion by the lichen phenols. This hypothesis would be in good agreement with Vesk *et al.* (1966) findings in manganese deficient plants, but the granal degeneration might be related to a water deficit as well, which is induced by the lichen extract (unpublished).

The increase in the number and size of plastoglobuli in a senescent chloroplast, that can be an accumulation of membrane breakdown products (Butler and Simon 1971), has also been described under stress conditions such as viral infection (Kolehmainen *et al.* 1965) and nutrient deficiencies (Vesk *et al.* 1966). In our assays, such an increase was also noticed, though it was not as high as other cases cited in the literature.

The peripherical display of thylakoids found in chloroplasts of explants of *Q. rotundifolia* treated with the lichen substances was associated with a stroma free from thylakoidal lamellae and rich in structures that in a transverse section looked like paracrystalline bodies and in a longitudinal view as fibrillar formations, somehow resembling the stroma-centres described by Gunning *et al.* (1968) as hexagonal packing.

Our observations differ from those of other investigators as far as size is concerned: The diameter of our hexagonal subunits, 10—11 nm, is over the 8.5 nm measured by Gunning *et al.* (1968) and below the 17—22 nm of Bartels and Weier (1967). It is in agreement with the size of the inner core of the circular subunits of the crystalline bundles found by Sprey (1968) in plastids of *Hordeum vulgare.* But Sprey (1968) sees an electron-dense core surrounded by an electron-transparent zone of 3.5 nm, while we see a transparent core and a dense cover. Such differences may be a consequence of fixation methods, or due to the high concentration of phenols in *Q. rotundifolia* leaves.

In spite of our scheme being somehow similar to that of Sprey, 10—11 nm in diameter and 13 nm between centres, we suppose the hexagonal shape of the subunits and we are not able to state precisely the fibrils size because in a longitudinal section they looked curved and it is not clear, if we are seeing them in full length.

Structures that may be related to ours have been observed closely associated to granal and intergranal membranes in *Macadamia* by Price and Thomson (1967) and also Sprey (1968) has reported in *Hordeum vulgare* a connection between fibrillar bundles and thylakoids in the phase of rapid greening. Price and Thomson (1967), Sprey (1968), and Gunning *et al.* (1968) relate this kind of structure functionally or developmentally to the photosynthetic membranes.

Sprey (1968) and Wrischer (1973) have demonstrated that fibrillar aggregates are formed from a pre-existing material, since their formation is not affected by protein synthesis inhibitors. The hypothesis that paracrystalline formations correspond to virus aggregates was discarded by proper assays at the beginning of our experiments. The structures also do not correspond to the ribulose 1,5-bisphosphate carboxylase, the appearance of which (Lanaras and Codd 1981) is different.

Having in mind the stearic continuity between the thylakoid lamellae and these formations and the presumable substitution of the former by the latter, we take the described structures as the result of photosynthetic lamellae degradation, in the same manner as similar structures have been associated with the neoformation of thylakoids. The chloroplast breakdown is obviously the reason for the lowered photosynthetic rate and further stimulation of senescence in Q. *rotundifolia* explants when incubated with *E. prunastri* lichen substances. We cannot conclude whether such phenolics are directly the inducers of the degenerative changes and the paracrystalline structures illustrated in this paper or whether they simply act as inducers of the senescence process.

REFERENCES

Asahina, Y., Kurokawa, S.: On the harmful effects of epiphytic lichens upon the higher plants. — Misc. Rep. Res. Inst. nat. Resour. (Tokyo) **25**: 83—86, 1952.

Bartels, P. G., Weier, T. E.: Particle arrangements in proplastids of *Triticum vulgare* L. seedlings. — J. Cell Biol. **33**: 243—253, 1967.

Brown, M. T., Mikola, P.: The influence of fruticose soil lichen upon the mycorrhizae and seedling growth of forest trees. — Acta forest. fen. **141**: 1—22, 1974.

Butler, R. D., Simon, E. W.: Ultrastructural aspects of senescence in plants. — In: Strehler, D. (ed.): Advances in Gerontological Research III. Pp. 73—123. Academic Press, New York—London 1971.

Dauriac, H., Rondon, Y.: Quelques aspects des propriétés d'un lichen: le *Letharia vulpina* (L
Hue en biologie experimentale. I. Action sur la germination du blé et le developpement
l'ail. — Bull. Soc. Bot. Fr. **123**: 235—241, 1976.

Diaz-Ruiz, J. R.: A highly ordered protein from *Pelargonium:* Structure and cellular localizatio
— J. Ultrastruct. Res. **53**: 227—234, 1975.

Dolzmann, P., Ullrich, H.: Einige Beobachtungen über Beziehungen zwischen Chloroplast
und Mitochondrien im Palisadenparenchym von *Phaseolus vulgaris.* — Z. Pflanzenphysi
55: 165—180, 1966.

Estévez, M. P., Vicente, C.: Inhibición por cloroatranorina de la reducción de NADP$^+$ p
ferredoxina de *Evernia prunastri.* [Inhibition by chloroatranorin of NADP$^+$ reduction f
Evernia prunastri ferredoxin.] — Bol. real. Soc. españ. Hist. Nat. (Biol.) **74**: 35—38, 1976.

Follman, G., Peters, R.: Flechtenstoffe und Bodenbildung. — Z. Naturforsch. **21*b***: 386—3
1966.

Gunning, B. E. S., Steer, M. W., Cochrane, M. P.: Occurrence, molecular structure and induc
formation of the stromacentre in plastids. — J. Cell Sci. **3**: 445—456, 1968.

Kolehmainen, L., Zech, H., Wettstein, D. von: The structure of cells during tobacco mos
virus reproduction. Mesophyll cells containing virus crystals. — J. Cell Biol. **25**: 77—97, 19

Lanaras, T., Codd, G. A.: Ribulose 1,5-bisphosphate carboxylase and polyhedral bodies
Chlorogloeopsis fritschii. — Planta **153**: 279—285, 1981.

Manocha, M. S., Shaw, M.: The physiology of host-parasite relations. XVI. Fine structure of
nucleus in the rust-infected mesophyll cells of wheat. — Can. J. Bot. **44**: 669—673, 1966.

Orús, M. I., Estévez, P., Vicente, C.: Manganese depletion in chloroplasts of *Quercus rotundif*
during chemical simulation of lichen epiphytic states. — Physiol. Plant. **52**: 263—266, 19

Price, J. L., Thomson, W. W.: Occurrence of a crystalline inclusion in the chloroplasts of *Ma
damia* leaves. — Nature **214**: 1148—1149, 1967.

Pyatt, F. B.: The inhibitory influence of *Peltigera canina* on the germination of graminace
seeds and the subsequent growth of the seedlings. — Bryologist **70**: 326—329, 1967.

Ramaut, J. L., Thonar, F.: Inhibition de la germination de differentes graines d'angiosper
par *Evernia prunastri* (L). Ach. — An. Quím. **68**: 575—595, 1971a.

Ramaut, J. L., Thonar, F.: Inhibition de la germination de differentes graines d'angiosper
par *Evernia prunastri* (L.) Ach. 2eme partie. — An. Quím. **68**: 597—607, 1971b.

Reynolds, S.: The use of lead citrate at high pH as an electron-opaque stain in electron microsc
— J. Cell Biol. **17**: 208—212, 1963.

Santesson, J.: Studies on the chemistry of lichens. **24**: Thin layer chromatography of aldeh
aromatic lichen substances. — Acta chim. scand. **19**: 2254—2256, 1965.

Shumway, L. K., Weier, T. E., Stocking, C. R.: Crystalline structures in *Vicia faba* chloropl
— Planta **76**: 182—189, 1967.

Sprey, B.: Zur Feinstruktur der Plastidenstromas von *Hordeum vulgare* L. — Protoplasma
469—479, 1968.

Spurr, A. R.: A low viscosity epoxy resin embedding medium for electron microscopy. —
Ultrastruct. Res. **26**: 31—43, 1969.

Staehelin, L. A.: Chloroplast fibrils linking the photosynthetic lamellae. — Nature **214**: 1
1967.

Teichler-Zallen, D.: The effect of manganese on chloroplast structure and photosynthetic ab
of *Chlamydomonas reinhardi.* — Plant Physiol. **44**: 701—710, 1969.

Thomson, W. W., Dugger, W. M., Palmer, R. L.: Effects of peroxyacetyl nitrate on ultrastruc
of chloroplasts. — Bot. Gaz. **126**: 66—72, 1965.

Thomson, W. W., Dugger, W. M., Palmer, R. L.: Effects of ozone on the fine structure of
palisade parenchyma cells of bean leaves. — Can. J. Bot. **44**: 1677—1682, 1966.

Vesk, M., Possingham, J. V., Mercer, F. V.: The effect of mineral nutrient deficiencies on the structure of the leaf cells of tomato, spinach and maize. — Aust. J. Bot. **14**: 1—18, 1966.

Vicente, C.: Aislamiento e identificación de las sustancias liquénicas. [Isolation and identification of lichen substances.] — In: Alhambra (ed.): Fisiología de las Sustancias Liquénicas. Pp. 43—70. Madrid 1975.

Wrischer, M.: Protein crystalloids in the stroma of bean plastids. — Protoplasma **77**: 141—150, 1973.

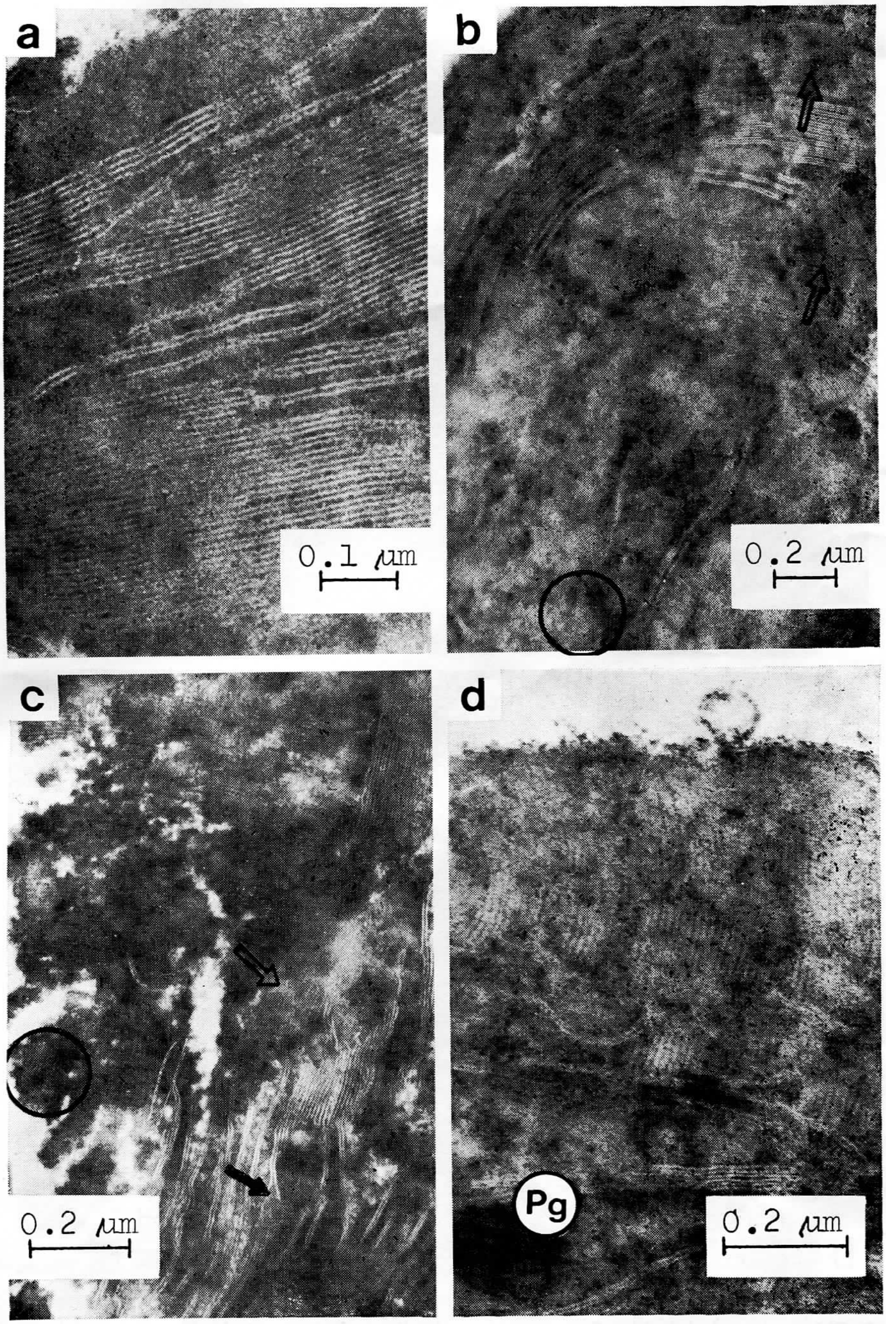

Fig. 1. Chloroplast thylakoids of control explants of *Quercus rotundifolia* (*a*) or explants treated with lichen substances (*b*) to (*d*) which show a highly disorganized structure. The display of lamellae is peripherical and widespread thylakoids look as if they are going to disappear (*white arrows*). Stromacentre-like structures lie in the stroma, where semi-crystalline bodies are also found (*circles*). In the next phase (*c*) thylakoids swell (*black arrows*) and disappear (*white arrow*). Stromacentre-like structures lying in the stroma are especially clear in (*d*), where also plastoglobuli (Pg) appear.

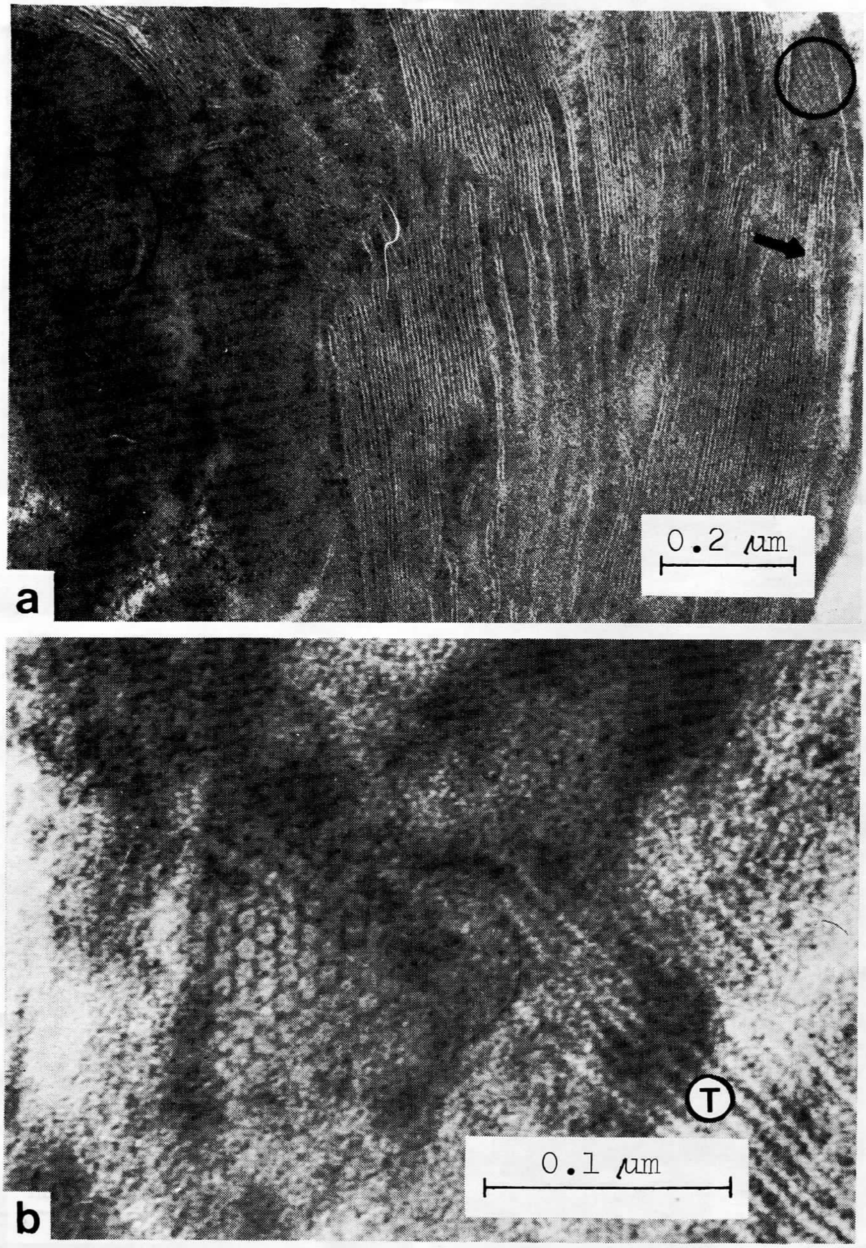

Fig. 2. Chloroplast thylakoids of *Quercus rotundifolia* explants treated with lichen substances (*a*) show a peripherical display of thylakoids, swelling thylakoids (*black arrow*), and semi-crystalline inclusions (*circles*; for details see *b*) occupying nearly all stroma free from thylakoids. *T*: thylakoid.